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THE CLINICAL SIGNIFICANCE OF BLOOD ERGOTHIONEINE

by

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THE UNIVERSITY OF ALBERTA

THE CLINICAL SIGNIFICANCE OF BLOOD ERGOTHIONEINE

A DISSERTATION
SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES
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INTRODUCTION

Since the establishment of the formula for ergothioneine, and the identification of it as an erythrocyte constituent (12, 20, 4, 13, 5, 6, 7) little work has been reported on values in normal and abnormal human blood. Interest was renewed with the reports of Lawson and Rimington (24, 25) in 1947 which attributed an antithyroid property to ergothioneine. Stanley and Astwood (28) and Wilson and McGinty (31) made attempts to confirm the antithyroid effect, both groups using radioactive iodine techniques, but they were unable to confirm the earlier reports of the London workers.

There then appeared to be good reason to investigate blood ergothioneine content in various disease states, and following the development by Hunter of a modification of his earlier diazo test (19, 21) this project was begun.

Salt (27) in 1931, using a modified uric acid method of estimation, reported slightly elevated blood ergothioneine levels in 17 cases of diabetes mellitus, and single cases of rheumatism, pulmonary tuberculosis, and hemiplegia. He found no variation of ergothioneine level with age or sex, and no correlation of the ergothioneine value in diabetes with the blood sugar levels, state of disease, or type of treatment. There was no relationship of ergothioneine level to blood uric acid

values, nor to the degree of urea retention. Benedict, Newton and Behre (4) had previously reported higher than normal levels in diabetes, but had given no values.

Latner and Mowbray (22), who used a colorimetric method developed by the former, (23) reported lower than normal blood ergothioneine levels in 4 cases of thyrotoxicosis, and low normal values in two others. They made the observation that ergothioneine, on addition to blood, was not combined with the blood plasma proteins, and could be easily and rapidly excreted by the kidneys. This they felt, might explain the failure of injected ergothioneine to act as an antithyroid substance.

Only two reports on the pharmacology of ergothioneine have been found. Tainter (29) in 1927, demonstrated no change in blood glucose levels or other signs of pharmacological activity in cats following the subcutaneous injection of ergothioneine. Trabucchi (30) an Italian worker, also considered ergothioneine inactive, but thought that it exhibited a mild anti beriberi action.

METHODS AND MATERIALS

Blood for estimation in diabetes was obtained from patients admitted to the Royal Alexandra and University Hospitals, either for periodic checks or because of a

diabetic complication. The samples were portions of oxalated blood taken by the hospital laboratory staff for blood glucose estimations.

Blood for estimation in tuberculosis was obtained from the Charles Camsell Hospital, in which case it came from either Indian or Eskimo patients, and from the Royal Alexandra Hospital, where all blood was taken from white patients.

Other diseases were investigated in a chance manner. The laboratory at the Royal Alexandra Hospital supplied us with blood remaining after requested tests had been performed.

Blood from normal persons was obtained from the University student population and from donors at the Red Cross Blood Donor Clinic, blood from the latter being taken at the conclusion of the donation.

The method for estimation was that described by Hunter (21) in 1949, with one modification. It was found that the plasma, which Hunter stated gave a yellow colour with the diazo test, contributed to the colorimetric value measured as ergothioneine in whole blood. The plasma value was independent of the amount of ergothioneine present and proportional to the haematocrit reading for the plasma in any specimen. It was found that the colour developed by pure plasma gave an "ergothioneine-equivalent" value of 1.0 mg.%

TABLE I

Three Different Corpuscle-Plasma Dilutions From the Same Blood

Ergothioneine mg.% whole blood	Haematocrit %	Ergothioneine mg.% corpuscles (calculated)	Ergothioneine mg.% whole blood less plasma effect.	Ergothioneine mg.% corpuscles less plasma effect
2.5	20	12.5	2.5 -(80% of 1) = 1.7	8.5
4.0	40	10.0	4.0 -(60% of 1) = 3.4	8.5
5.5	60	9.2	5.5 -(40% of 1) = 5.1	8.5

TABLE 2

Comparison of Ergothioneine Values Found in Corpuscles and Calculated From Whole Blood

Ergothioneine mg.% whole blood	Haematocrit %	Ergothioneine mg.% corpuscles (Calculated)	Ergothioneine mg.% whole blood less plasma effect	Ergothioneine mg.% corpuscles (found)
3.3	42.5	6.4	3.3 -(57.5% of 1) = 2.7	6.4
2.2	46.0	3.7	2.2 -(54% of 1) = 1.7	3.7

± 0.2. A fraction of an average value of 1.0 mg.%, proportional to the percentage of plasma found in each specimen, was subtracted from the ergothioneine value obtained for the whole blood in the same specimen. This then gave the true ergothioneine reading.

Different dilutions of cells with plasma were made from one blood, and the above method of calculation for true ergothioneine content was found to be valid for all haematocrit values encountered. (Table I). This plasma correction is especially significant when the haematocrit is low, since the difference is increased to a greater extent on conversion of the ergothioneine reading from whole blood to per cent corpuscles. No ergothioneine was found in plasma, and the "ergothioneine-equivalent" readings in plasma remained constant in bloods which gave ergothioneine values ranging from 4 to 60 mg.% corpuscles. To confirm the belief that all the ergothioneine measured by this method was contained in the corpuscles, estimations were made on whole blood and the values converted by haematocrit to per cent corpuscles; estimations were then made on the corpuscles alone from the same sample. Two examples of this calculation are given in Table 2 in which it can be seen that the calculated value of ergothioneine per cent corpuscles using whole blood agrees with the value found on using corpuscles alone*

(* Footnote on page 6)

If the plasma effect was not taken into consideration, the respective values per cent corpuscles would be 7.8 mg.%, and 4.8 mg.%, readings 1.4 mg.% and 1.1 mg.% higher than the actual value for corpuscles of 6.4 mg.% and 3.7 mg.%.

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*To minimize the difficulty experienced in obtaining the corpuscles free from plasma dilution, the sample was first centrifuged at 3000 r.p.m. and then frozen. The erythrocyte layer was then cut from the frozen core, melted, and carried through the test. Freezing did not affect the test.

PROBLEMS CONNECTED WITH THE DIAZO TEST

To better explain the steps referred to in the following section a brief summary of the procedure of the diazo test is now given (21).

1. 0.5 ml. whole blood + 4.5 ml. of 0.0045 N acetic acid
2. Heat in a slowly boiling water-bath for 3-4 min.
3. Centrifuge and decant the supernatant liquid.
4. Supernatant + 1 drop of Goulard's solution (lead monoxide and lead acetate)
5. Centrifuge and decant the supernatant.
6. Supernatant + 1 drop of $NaH_2PO_4.H_2O$.
7. Centrifuge and decant the supernatant.
8. Add 2 ml. of buffer (sodium carbonate and sodium acetate) to 1 ml. of diazo (Koessler and Hanke) reagent in each of two colorimeter tubes.
9. To No.8 in 15 seconds add, in one colorimeter tube, 1 ml. of No.7 + 1 ml. of water, and in the second colorimeter tube 2 ml. of No.7.
10. To the coupled compound from No.9 in each colorimeter tube, add, in 45 seconds 5 ml. of 10 N NaOH.

This then gives the final violet-pink colour typical of ergothioneine.

a. Interference - definition and description.

In the estimation of blood ergothioneine a bronze and less commonly and orange-pink colour appears upon the addition of 10 N NaOH to the coupled compound, instead of the expected violet-pink colour. This interferes with the estimation of ergothioneine. Throughout this clinical investigation this problem has been particularly troublesome. Investigation into the mechanism of its production

has been sporadic both because of the limiting amount of blood which remains after the initial estimation, and because of our desire to accumulate sufficient data from disease cases for a statistical analysis, rather than any wish to examine the test itself.

In the routine of the test 1 and 2 ml. aliquots from the final protein-free filtrate (stage 7) are added to the diazo solution. In cases in which interference develops we have noticed nothing unusual in the colour of the coupled compound. However, upon addition of the 10 N NaOH which normally produces a violet-pink colour, the specimens with interference have developed one of two colours, an orange-pink or bronze. These foreign colours have inevitably been more marked in the 2 ml. aliquot; and in fact the 1 ml. sample has frequently given a good "ergothioneine" colour with proportionality between it and the 0.5 ml. aliquot in spite of an interfering colour in the 2 ml. aliquot. This freedom from interference in the smaller aliquots has been more common when the interference has been of the "orange" variety.

As the time of storage is increased the interfering colour which develops changes from orange to bronze; this latter colour appears to be the end point of the change. When such a colour is produced on testing each of the three aliquots (0.5, 1 and 2 ml.) it is impossible to make any estimation of the ergothioneine content.

In general, the "orange" interference has been found to give an apparent increase in the value read as ergothioneine and the bronze to produce a reduction in the true value. A blood specimen which has once developed an interfering colour consistently does so in that particular specimen.

b. Factors in the Production of Interference.

Several factors have been found to influence the development of interference.

1. Hemolysis

The appearance of interference is much more frequent in hemolysed specimens, and often develops in specimens stored at room temperature which initially gave good tests but which have become hemolysed.

2. Temperature

Duplicate quantities from the same specimen have been refrigerated and stored at room temperature. Those at room temperature developed interference in 0-2 days, whereas those which were refrigerated could frequently be kept for as long as 10 days.

3. Time

It has been found that the less the time that elapses from venepuncture to test, the less the incidence of interference.

4. Disease

Blood from both normal and diseased persons may dev-

elop interference, but we have observed a high incidence of it in our series in blood from patients who were suffering from two disease states - kidney failure (nephritis) and leukemia (but not two cases of aleukemic leukemia).

c. Mechanism of Interference.

Much of the investigation into the development of interference has produced equivocal results, but various possibilities which have been examined are presented here to illustrate the state of the problem to date.

(i) pH of the Blood

Readings of the Beckman pH meter gave a mean reading of 6.88 for 3 blood specimens which had given good ergothioneine colours and an identical mean of 6.88 for four which had shown interference.

(ii) Coagulation Heating Time

By varying the time that the blood-acetic acid solution was in the water-bath from 1 to 15 min., we attempted to assess the effect on the production of interference. In one specimen interference was present in the filtrate from the portion heated 8 min., but not in that heated 2 min. This isolated finding has not been repeated.

(iii) Source and Mode of Production of Interference

In an attempt to determine whether the interfering substance was present in a) the plasma, or b) the erythrocytes, or c) was dependent upon some interaction

between these two phases, the following experiment was planned.

i. To test plasma from cases showing interference. "Ergothioneine-equivalent"* values for these did not differ significantly from plasma values for specimens without interference.

ii. To add plasma from a case showing interference to erythrocytes from blood showing no interference. The value for a 50% mixture of plasma, from a specimen showing interference, and cells, from blood developing a normal ergothioneine colour, when corrected to mg.% corpuscles, was equal to the original value for the cells used. This was repeated on two more different plasma-cell mixtures with the same results. In contrast to these results, in another specimen, washed cells** from a case of interference gave a good ergothioneine colour but upon readdition of the original plasma, interference again appeared, although to a lesser degree than in the whole blood.

To see if a higher concentration of plasma would produce different results, a 75% - 25% mixture of plasma from a specimen with interference and cells from blood giving a good ergothioneine colour was made. A typical interference developed in the 2 ml. aliquot, but the 0.5 and 1 ml. aliquots gave readings close to the original value for the cells used. This would indicate that by decreasing the concentration of the cells the effect

(See footnotes on bottom of next page)

of the interfering substance is increased, and that the interfering material is present in the plasma.

Cells from another sample showing interference were separated and washed four times in 1% saline. These then gave a good ergothioneine colour with no sign of interference. In order to see if the production of the interfering material took place during the hemolysis and heating (step 2) - that is, through some plasma-erythrocyte reaction - the separate filtrates from the plasma and the washed cell fractions of the same sample were carried through to stage 7, then mixed, in equal quantities. The test on this filtrate mixture showed the previous interference colour found in the whole blood of the same specimen.

The following conclusions could then be drawn:

1. The interfering substance or an essential constituent of it is contained in the plasma.
2. The formation of it does not depend on any reaction of a plasma factor with the red cell fraction during hemolysis and heating (stage 2).

In an effort to identify the substance a paper chromatogram was very kindly done for us by Mr. K.A. Siluch of this Department on the filtrate from a specimen which

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*The value read in mgm.% ergothioneine, but not due to the colour found normally with ergothioneine.

** This procedure is discussed later.

showed typical bronze interference. He reported the presence of ergothioneine (not detected by colorimetry because of the interference), and a large stationary area which he suggested might have been due to the interfering material, but which he was unable to identify.

d. Possible Causes of Interference.

i. Contaminants.

Because interference has been shown to develop in all blood samples if allowed to stand long enough, contaminants are unlikely causes of interference. Some of these were investigated.

"Hemosol"

This is a solvent of unknown composition, used, in both hospitals from which the blood was obtained, for cleaning test tubes (R.A.H. and U.H.). When added to 4 samples it caused no interference.

Acid-chromate Cleaning Solution.

This was used in cleaining our own equipment. Small amounts were added to the diazo reagent and gave rise to a greenish-yellow colour. However, when added to blood which was then carried through the test it produced no interfering colour.

ii. Physiological Substances.

Phenols.

Because of the high frequency of interference in

cases of uraemia it occurred to us that the phenols and phenol-like materials, the blood levels of which are said to parallel the uraemic manifestations (8), might be a cause. Within the limitations of the test with Millon's reagent (14) we were unable to demonstrate the presence of any phenols in the specimens showing interference.

Andrewes, as quoted by Cantarow and Trumper (16), found that on performing the indirect van den Bergh test on serum from patients with nephritis an orange-buff colour developed, which changed to cherry-red on addition of strong sodium hydroxide. Again because of the frequency of interference in cases of renal failure, we felt that it was possible that a similar substance might be responsible for the interference in the diazo test for ergothioneine. We would then have to assume the appearance of this same substance in the non-uraemic blood on storage. To try this possibility we applied the Andrewes test as modified by Hewitt (16), to the serum of blood samples showing interference. We were unable to demonstrate any positive tests except in blood from cases of uraemia.

Lactic Acid.

In many cases the time taken for the development of interference coincided with the time necessary for the completion of glycolysis and the exhaustion of the supply of co-enzyme 1 and diphosphopyridine nucleotide

(11). For this reason the effect of lactic acid, which accumulates with the cessation of glycolysis (11), was investigated. Lactic acid added to the diazo reagent gave no colour.

TABLE 3

The effect of different concentrations of lactic acid added to a normal blood (heated 4 min. stage 2)

Final Concentration of Lactic Acid			
0 mg.%	100 mg.%	200 mg.%	300 mg.%
0	0	+*	0

TABLE 4

The effect of different heating times on two blood samples with a final concentration 200 mg.% lactic acid

	1.5 min.	2 min.	3 min.	4 min.	6 min.
Blood A.	+	0	-	+	+
Blood B.	0	-	0	-	+

*0 - No interference.
 + - Interference present
+0 - An early interference colour with little change in value. These symbols will be the ones used in this and in subsequent tables.

Aliquots of Blood B were heated without lactic acid for 1, 1 1/2, 2, 3, 4, 5, 6 (twice), 7, 8, 9 and 10 min. periods. One of the aliquots heated 6 mins. showed interference; none of the other aliquots showed interference. These are representative results obtained in a number of similar experiments, and it can be seen that we were unable

to draw any conclusions from these findings.

Enzymes

Interference, in most specimens, is not initially present, but appears as the age of the blood increases. This fact suggested to us the possibility of being able to inhibit some enzymatic process and so prevent the development of a hypothetical product of an enzyme reaction, which could conceivably be the cause of interference.

Two enzyme inhibitors were used: heat and sodium fluoride. Five aliquots of blood were taken, from each of two specimens. One aliquot of each was kept refrigerated and one was kept at 37°C; the third and fourth were first heated at 100°C. for 0.5 mins. and 2 mins. respectively then kept at 37°C.; 1% sodium fluoride was added to the fifth which was then also kept at 37°C. (Table 5)

In both A and B samples refrigeration apparently delayed the appearance of interference. In neither sample did fluoride prevent the development of interference by the seventh day. Heating prior to incubation had no delaying effect in B blood; in fact the samples heated for 2 min. developed interference at an earlier date than the control. Heating both for 0.5 and 2 mins. was associated in sample A with a somewhat later development of interference than found in the control.

TABLE 5

The effect of enzyme inhibition on the rate of development of interference in normal blood.

Day of test	Kept at 37°C.		Refrigerated		Heated for 0.5 min.		Heated for 2 min.		Sod. fluoride added	
	Blood		Blood		Blood		Blood		Blood	
	A	B	A	B	A	B	A	B	A	B
0	0	-	-	-	0	0	0	0	0	0
1	+	0	0	0	-	0	0	0	-	-
4	+	0	-	-	0	0	+	+	-	-
7	+	+	0+	0	+	+	+	+	+	+

Because we have no idea what enzyme, if any, is involved, we have no way of choosing a suitable enzyme inhibitor, and it must be remembered that fluoride may have no effect on the system in question. Two further specimens were treated with fluoride and at the end of six days at 37°C. the fluoride treated samples showed the same amount of interference as the controls.

In this type of experiment it might be argued that bacterial growth must be considered. Because there is no apparent difference in the interference which is seen early, as for example in most blood from cases of nephritis and leukemia, or at various later times in other blood from normal persons, we have made no attempt to consider this aspect.

Glucose.

Because of the previously mentioned coincidence in the time it takes for failure of the glycolytic activity in stored blood and the lapse of time found in normal blood before the development of interference, we attempted to delay the termination of glycolysis. This activity can be prolonged by the addition of glucose to the specimen (11). This was done in two samples, and the time taken for the development of interference compared to two controls. The control in both samples showed interference in two days. One test sample with 400 mg.% glucose added,

gave a reasonably good colour, though slightly orange, in that time. The second sample displayed a fairly good ergothioneine colour in the 1 ml. aliquot but interference in the 2 ml., in comparison with its control which showed complete interference. (1 ml. and 2 ml.).

To see if the action of glucose in delaying interference could be attributed to its effect on either the plasma or the corpuscles, corpuscles and plasma were first separated, and each fraction divided into three aliquots. Glucose was added (400 mg.%) to one aliquot of cells and to one aliquot of plasma. The separate aliquots, including one pair as a control, were then kept at 37°C. for two days, then reconstituted, giving the following mixtures:

1. Corpuscles and plasma.
2. Corpuscles and plasma with added glucose.
3. Corpuscles with added glucose and plasma.

All three showed an orange-yellow colour with the diazo test, but the colour was somewhat more intense in the third sample. Glucose in this concentration increased, rather than decreased, the intensity of interference in the third combination. This avenue of attack on the problem seemed to offer nothing.

Amino Acids.

In searching for a common factor which might be responsible for interference in the two diseases, neph-

ritis and leukemia, it was noted that in both conditions blood amino acids are said to be increased, (1). In leukemia the increase is said to parallel the increase in leukocytes, a fact which would explain the absence of interference in two cases of aleukemic leukemia.

Histidine, histamine, tyrosine and a commercial preparation of amino acids ("Parenamine", Winthrop Stearns) were all added to blood samples to assess their effect.

TABLE 6

The effect on blood ergothioneine values of adding certain amino acids or derivatives

		Final concentration of added substance				
		0 mg.%	1 mg.%	5 mg.%	10 mg.%	15 mg.%
Histidine	1 ml.*	8.9	10.1	11.3	13.6	14.4
Blood A	2 ml.	8.8	9.8	9.8	12.7	14.9
Histamine	1 ml.	8.9	10.5	11.5	----	13.1
Blood A	2 ml.	8.8	10.4	11.7		13.5
Tyrosine	1 ml.	1.7	2.2	2.4	----	3.8
Blood B	2 ml.	1.3	2.0	2.2		3.9

*Volume of aliquot used (stage 9)

Histidine and histamine gave a similar burnt-orange colour to the end product of the test. This colour deepened in proportion to the amount of amino acid added, and did not resemble the typical interference colour.

Tyrosine produced a pinkish colour, but not the violet-pink of ergothioneine. It also increased in proportion to concentration.

Glutamic acid was also added at this time but appeared to have no effect on the development of the normal ergothioneine colour in blood.

The task of adding individual amino acids appeared formidable, and the conditions obtained from introducing single amino acids were probably not comparable to those found when combinations of acids were present, as is the case in blood. For these reasons various amounts of a commercial amino acid preparation ("Parenamine", Winthrop-Stearns) were added in the belief that this might give a picture somewhat more like a physiological increase.

TABLE 7

The effect on ergothioneine reading of adding different amounts of "Parenamine" to blood samples

	Final concentration of amino acids 0 mg.%	 50 mg.%	 100 mg.%*
Aliquots 1 ml.	3.8	4.7	4.7
2 ml.	3.7	4.7	4.8

*100 mg.% "Parenamine" is equivalent to 13.3 mg.% expressed as amino acid nitrogen.

The "Parenamine" also caused an increase in the value read as ergothioneine giving a slightly orange-

pink colour, again not the colour of natural interference.

Samples of blood to which amino acids ("Parenamine") were added were incubated and the following results noted:

TABLE 8

The effect of "Parenamine" on the development of interference in blood samples kept at 37°C.

	Day 1		Day 2		Day 3	
	Control	Test*	Control	Test	Control	Test
Blood E	0	0	+0	+0	+	+
Blood F	0	0	0	+0	+	+

*Parenamine added to give a concentration of 25 mg.% amino acid equivalent to approximately 3.3 mg.% as nitrogen.

In blood sample F there was a slightly earlier appearance of an interfering colour in the test sample with the added amino acids. There appeared to be no significant decrease in the time needed for the development of interference when amino acids were added to human blood.

Iron.

Barger and Ewins (2) showed that the sulphur of the thiolimidazole ring of ergothioneine is oxidized and removed in the presence of ferric iron. Iron was

tested as a possible cause of interference because of this reputed ability to destroy the thiolimidazole ring.

Sufficient ferric chloride was added to a blood sample which gave a good ergothioneine test to give a resulting concentration of 52 mg.% ferric iron. The result was the production of a bronze colour. Amounts were then added as shown in the following table.

TABLE 9

The effect on ergothioneine reading of adding different amounts of ferric chloride to blood samples

		Final concentration of ferric iron			
		0 mg.%	1 mg.%	10 mg.%	25 mg.%
Blood A		(+)	(0)	(+0)	(+)
	1 ml.*	3.5	3.6**	3.8	3.8
	2 ml.	3.5	3.0	3.4	2.8
Blood B		(0)	(0)	(0)	(0)
	1 ml.	4.7	4.7	4.9	5.1
	2 ml.	4.8	4.3	5.1	5.0

*Volume of aliquot used (stage 9)
**Ergothioneine mg.% corpuscles

It was interesting to note in Blood A the change from the orange interference found in blood with 10 mg.% iron through the orange-bronze of the blood with 25 mg.% to the final definite bronze of the blood with 52 mg.% iron. This is the same sequence of colour change with passage of time which we find in normal blood standing at room temperature. Blood B presents one of the puzzling inconsistencies which characterizes much of

this work on the problem of interference.

Ascorbic Acid.

In the search for a physiological component of blood, which might be responsible for interference, ascorbic acid was investigated because of its high value in leucocytes, which is approximately 40 times that of erythrocytes (18), and the coincident frequency of interference in leukemia.

Greenblatt (15) has shown that the imidazole ring of ergothioneine is split by ascorbic acid in vitro. He also showed that ascorbic acid given intravenously to guinea pigs, rabbits and human subjects, caused no change in blood ergothioneine levels. Barron and Kemperer (3) stated that the presence of red blood cells had no effect on the rate of oxidation of ascorbic acid in blood or in serum. They suggested that the main protection was provided by proteins, amino acids and glutathione. Contradictory results are presented by Heinemann (18) who showed that ascorbic acid oxidation proceeded more rapidly in serum than in blood, and more rapidly yet in a mixture of serum and leukocytes. The presence of intact red cells in any one of these mixtures markedly inhibited oxidation. Oxidation was dependent upon access to atmospheric oxygen and did not take place when any one of the solutions was placed in an atmosphere of

nitrogen.

At the pH and temperature of tissues the dehydroascorbic acid formed on oxidation of ascorbic acid undergoes, in a few minutes, an irreversible change to another acid, ketogulonic, which has more reducing power than reduced ascorbic, and which then gives rise to undetermined products, perhaps oxalic and l-threonic acids, (9, 26).

Red blood cells are virtually impermeable to ascorbic acid (18, 9). This suggested to us that some interaction might be postulated to occur between ascorbic acid and products formed during erythrocyte hemolysis, during the heating stage of the test. Such a reaction is unlikely since we later found that the reconstituted plasma and washed-erythrocyte filtrates from a sample of blood which previously showed interference still produced a bronze colour.

Ascorbic acid, both in the reduced and in the oxidized form, when added in aqueous solution to the diazo reagent, gave a very pale yellow-green colour. Oxidized and reduced ascorbic acid in amounts sufficient to give final concentrations of 5 and 25 mg.% in both cases, were added to aliquots of blood which gave a normal ergothioneine colour. Neither the oxidized nor the reduced form produced any change in the usual colour of ergothioneine. When added, however, to the filtrate

of any sample at either stage 3 or stage 7, both reduced and oxidized ascorbic acid prevented the development of the ergothioneine colour and gave rise to a bronze,indistinguishable from the natural interference colour.

Reduced and oxidized ascorbic acid were added to aqueous solutions of ergothioneine,and the resultant mixtures carried through the complete test. When either reduced or oxidized ascorbic acid was added to give a concentration of 5 mg.%,a normal ergothioneine colour developed. However in the test solution containing a concentration of 25 mg.% reduced ascorbic acid,the colour developed was orange-pink, and in the solution containing 25 mg.% oxidized ascorbic acid the colour was bronze. No reduced ascorbic acid was found in the final filtrates of any of these four tests.

Both oxidized and reduced ascorbic acid were added at two other stages in the procedure; before the addition of Goulard's solution (stage 3) and after the addition of sodium acid phosphate (stage 7). Both in concentrations of 5 and 25 mg.%, and when added at either stage 3 or stage 7, both of these substances interfered with the production of a normal ergothioneine colour. In every case the final colour was bronze.

The coupling colour (produced in stage 9 of the

test) in these cases of induced interference was normal. It therefore appeared likely that the ascorbic acid or its product did not affect the coupling of the ergothioneine with the diazo reagent, but rather modified the ergothioneine molecule. For this reason ascorbic acid was added, both in the reduced and oxidized state, to the reaction mixture during the 45 second interval between coupling and the addition of sodium hydroxide. This also produced the same bronze interference-like colour.

Final filtrates of blood samples to which reduced ascorbic acid had been added were tested by the sodium 2-6 dichlor-phenol-indo-phenol method and no reduced ascorbic acid was ever found. Similarly filtrates from specimens with both interference and normal colour gave no positive tests. The passage of reduced ascorbic acid through the heating stage had been shown, however to be unnecessary for the production of an ascorbic acid induced interference (cf. aqueous solution of ergothioneine with added reduced ascorbic acid, above). It is questionable whether either form of ascorbic acid reaches the final filtrate in the ordinary test procedure because ascorbic acid is precipitated at pH 7.6 by lead (16). This is somewhat more alkaline than the corresponding pH in the procedure, and we are unable

to say whether oxidized ascorbic acid reaches the final filtrate. The fact that the oxidized form of ascorbic acid causes interference when added to an aqueous solution of ergothioneine prior to the heating stage yet neither the reduced nor the oxidized form causes interference when added to normal blood,indicates that blood exerts some modifying influence on the ascorbic acid-ergothioneine relationship.

Experiments were then designed to see if an excess of ascorbic acid added to normal blood would hasten the appearance of interference at 37°C.

TABLE 10

The effect of added ascorbic acid on the rate of development of interference in normal blood.

	Day 1		Day 2		Day 3	
	Control	Test*	Control	Test	Control	Test
Blood A	0	0	0	0	-	-
Blood B	0	0	0	+	-	-
Blood C	0	0	+0	+0	+	+
Blood D	0	0	0	0	+	+

*Sufficient ascorbic acid was added to give a concentration of 5 mg.%.

Blood B in Table 10 was the only one in which addition of ascorbic acid in the concentration used appeared to hasten the development of an interfering colour.

It was possible that amino acids might exert some influence on the effect of ascorbic acid. These were now added together.

TABLE 11

The effect of a combination of amino acids and ascorbic acid on the rate of development of interference in normal blood.

	Day 1		Day 2		Day 3	
	Control	Test*	Control	Test	Control	Test
Blood C	0	+0	+0	+	+	+
Blood D	0	+0	0	+0	+	+0

*Parenamine and ascorbic acid added to give final concentrations of 25 and 5 mg.% respectively. Incubated at 37°C.

In Blood C (Table 11) interfering colour appeared one day earlier in the test aliquot. In Blood D an"early" interfering colour (orange-pink) appeared two days earlier than in the control, but never developed into the final bronze colour seen in the control on the third day. There does not seem to be any significant difference between these results and those found on addition of amino acids alone.

It was now reasonably established that ascorbic acid could, in reduced or oxidized state, interfere with the ergothioneine diazo reaction; that it could probably do so through virtue of its ability to modify the ergothioneine imidazole ring rather than to interfere with

the coupling reaction; and that there is some property of whole blood which normally prevents ascorbic acid causing this interference.

e. Methods of Preventing and Reversing Interference.

Prevention.

This may be accomplished by:

i. Using the blood as soon as possible after venepuncture.
ii. Refrigerating the blood.
iii. Avoiding hemolysis.
iv. Not allowing the oxalate concentration to exceed 2 mg. per ml. blood.

In taking duplicate specimens in test tubes used by the hospital laboratory staff and in our own, we found interference in two of the former, but in neither of the latter. Reduction of the oxalate content of the hospital tubes from 4 to 2 mg. per ml. resulted in a marked decrease in the number of samples showing interference. Specimens with the higher oxalate concentration also showed slight hemolysis, a factor already discussed in relation to interference.

Blood was taken from two normal students, A and B, and oxalated with 2, 4 and 8 mg.% per ml. These samples were then incubated for a 5 day period.

TABLE 12

The effect of different oxalate concentrations on the rate of development of interference in normal blood*

	Oxalate 2 mg./ml.		Oxalate 4 mg./ml.		Oxalate 8 mg./ml.	
	A	B	A	B	A	B
Day 0	-	0	-	-	0	-
Day 2	0	0	0	0	+	+
Day 3	0	-	0	0	+0	0

*Incubated at 37°C.

It will be seen that with 8 mg. of oxalate per ml. blood, interference appeared in both A and B blood on the second day. The puzzling finding was the lack of interference in B when repeated on the third day. The fact remains that much better results have been obtained since the reduction of oxalate content.

Reversal of Interference.

In this section we will also mention for the sake of record, the following method which was used unsuccessfully in an attempt to get rid of the interfering colour.

Uricase.

Hunter (21) suggested that combined uric acid might be the cause of interference in ox blood, and possibly human blood. An isolated sample of ox blood

gave an ergothioneine colour following incubation with the enzyme uricase (21). This suggested to us the possibility of treating our human blood showing interference in the same manner. In two samples this treatment had no apparent effect on the bronze colour.

1. Freezing.

This was the first method by which we were able to obtain a good colour on a specimen which otherwise showed interference. It appears to work only by virtue of the separation of the plasma from the erythrocyte fraction. Because of what may have been an adsorption of the interfering material on the outside of the red cells, the separation was not always complete, and interfering colour still remained in a considerable number of the red cell fractions.

2. Washing Red Cell Fraction.

We then attempted to rid the red blood cells more completely of plasma by washing them four times in 1% saline, discarding each washing. The cells, suspended in a 1% saline solution to give about a 50% concentration of cells, were then carried through the test. It was found that precipitation was not satisfactory unless a weaker acetic acid was used. We substituted 0.0020 N acetic acid for the usual 0.0030 N strength

and obtained good results.

With the adoption of this method we found that it was possible to get rid of interference in the majority of cases. To see if these was any loss of ergothioneine in the course of this procedure, the following test was run. One sample which gave a value of 6.9 mg.% corpuscles was allowed to develop interference. When the red blood cells were washed,a good ergothioneine colour was again observed with a reading of 7.0 mg.% corpuscles, checking closely with the original known value of 6.9 mg.%.

Some of the values found in the blood samples with interference, both before and after the saline-washing procedure, are shown in Table 13. It will be seen that there was no consistent relationship between the values for the washed cells and the whole blood. The final colour in all washed cell samples was the violet-pink of ergothioneine, and we felt confident that the reading obtained was reliable in each case. Haematocrit values were found for the suspensions of red blood cells in saline and the values per cent corpuscles were calculated.

Samples showing interference may or may not exhibit proportionality between the 1 and 2 ml. aliquots. The interfering colour may cause an apparent increase or decrease in the true ergothioneine reading. No

TABLE 13

Ergothioneine values in blood showing interference, before and after saline washing.

	Whole Blood with Interference			Washed Red Blood Cells		
	Ergothioneine mg.% blood	Haemat-ocrit	Ergothioneine mg.% corpuscles	Ergothioneine mg.% suspension	Haemat-ocrit	Ergothioneine mg.% corpuscles
Blood A.	5.1* 3.2**	27.5	18.0	7.0 7.2	46.5	15.3
Blood B.	3.4 3.9	23.0	11.9	4.0 3.8	24.0	16.4
Blood C.	3.1 3.3	39.0	6.6	3.5 3.5	31.0	11.3
Blood D.	3.7 3.6	33.5	8.9	7.3 7.1	46.5	15.5
Blood E.	1.4 1.4	36.0	2.0	2.2 2.0	52.5	4.0
Blood F.	10.8 4.3	39.0	31.4	5.0 5.2	25.0	20.4
Blood G.	9.0 6.1	34.5	27.1	6.8 6.8	28.5	23.8
Blood H.	2.8 2.5	35.0	5.7	3.4 3.4	17.5	19.4

*1 ml., **2 ml. aliquots of filtrate used for each of these readings respectively.

estimate can be made of the degree of direction of correction from the resulting bronze colour in a sample with interference. It should also be stated that we have found the washing method (for getting rid of interference) is not always successful when used on a blood with initial interference, which has been allowed to stand for some time. This procedure is also obviously inapplicable to any specimen which is hemolysed on receipt.

In summary, the following observations have been made:

1. Although we have not yet established the identity of the interfering material, we have discovered several factors associated with its production.
2. Ascorbic acid is a possible cause. Ascorbic acid or one of its degradation products may act on the imidazole ring of ergothioneine, or in some undetermined way modify the ergothioneine structure.
3. Several suggestions are offered for reducing the incidence of interference in blood samples.
4. In most cases which have developed even complete interference a reliable value can be established by the saline-washing technique.

DISEASES INVESTIGATED

DIABETES MELLITUS

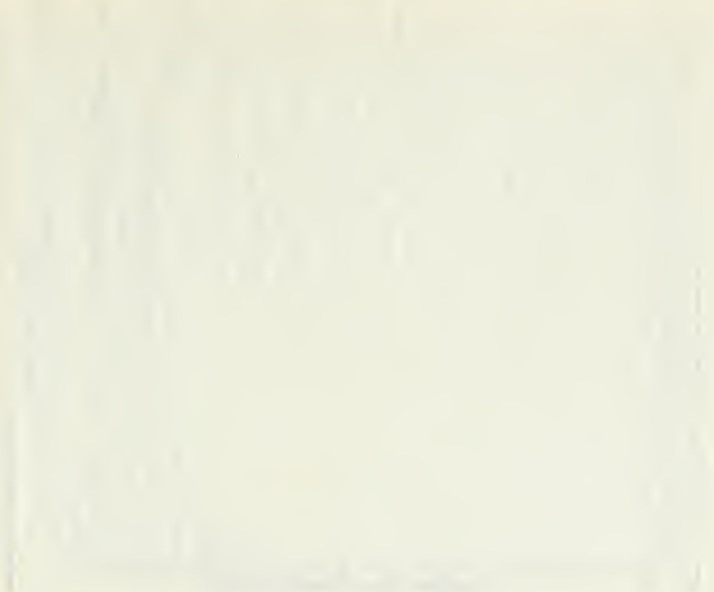
The diabetic group of 107 hospital patients was compared with the normal group of 94 persons. 50.9% of the diabetics were males and 49.1% females; 80.8% of the normal group were males and 19.2% females. A comparison of the two groups is shown in Table 14.

TABLE 14

Ergothionesine values in normal and diabetic blood

	Normal	Diabetic
Number of patients	94	107
Average age (years)	29.5	52.1
Average age - males	29.6	52.7
Average age - females	29.1	52.2
Mean ergothioneine value mg.% corpuscles for males and females	9.6	12.7
Mean - males	9.7	13.0
Mean - females	9.5	12.2
Standard error of mean	0.32	0.80
t value found = 3.580		
t value at 1% level = 2.601		

On statistical comparison of the two means a highly significant difference was found to exist between the normal and the diabetic groups.



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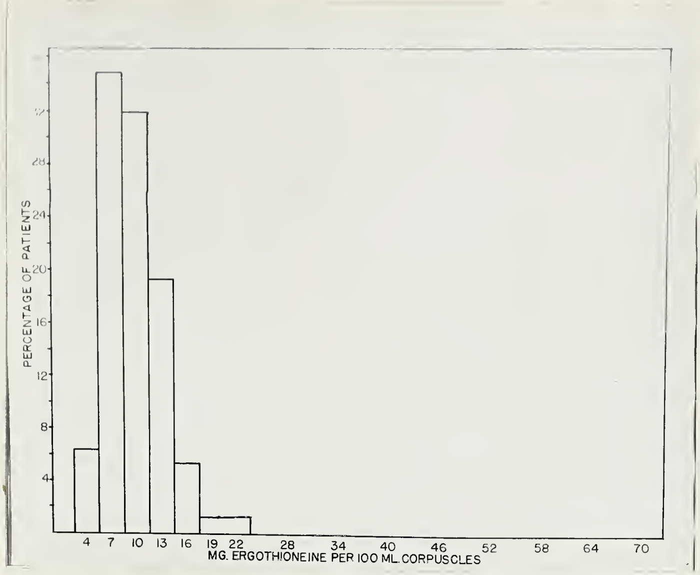

Figure 1 Distribution of blood ergothioneine levels of 94 normal subjects.

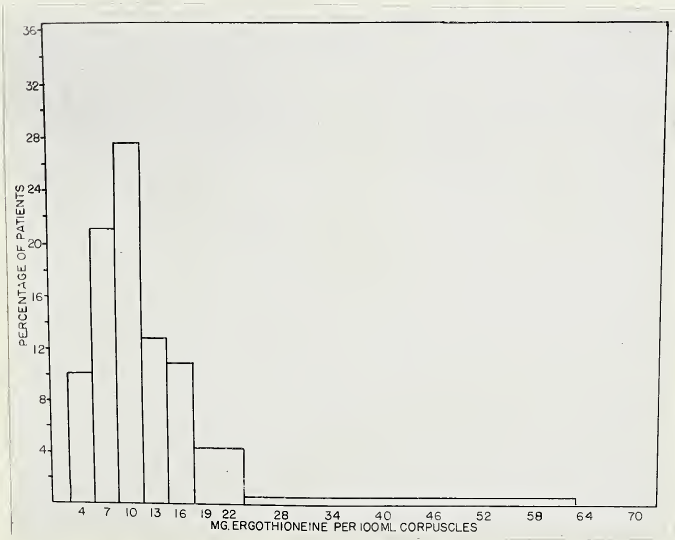

Figure 2 Distribution of blood ergothioneine levels of 107 diabetic patients.

It will be seen from Figure 2 that this difference was caused by diabetics who had ergothioneine values greater than 14.5 mg.%. No normal person had a value greater than 17.7 mg.%. The range for normals extended from 3.9 to 17.7 mg.% and the range for diabetics from 2.0 to 60.2 mg.%. Seventeen diabetic patients (15.9% of the total number of diabetics) exceeded the highest normal value.

Because the mean of the age of the normal group was much lower than that of the diabetic series it was felt that a comparison of ergothioneine levels in different age groups in the normal should be made.

TABLE 15

Blood ergothioneine levels at different age intervals in normal persons

Age Interval years	Number of Persons	Ergothioneine mg.% corpuscles	Standard error,mean
15 - 19	5	8.3	1.71
20 - 24	24	9.7	0.52
25 - 29	38	9.00	0.37
30 - 34	9	11.2	1.02
35 - 44	5	7.7	0.40
45 - 63	13	11.6	1.17
25 - 29*	38	9.0	0.37
30 - 63*	27	10.7	0.70

*t value found = 0.659
t value at 5% level = 2.000

It will be seen in Table 15 that the ergothioneine value in the 45 - 63 year interval was greater than the mean for the group as a whole. The increase was not progressive throughout the group, however, and on comparison of two approximately equal number of patients from the 25 - 29 year interval (38 patients) and the 30 - 63 year interval (27 patients) we found no significant difference between the means.

The diabetic series was then divided into age groups, and in contrast to the normal group, showed a somewhat lower value in the older age intervals.

TABLE 16

Blood ergothioneine levels at different age intervals in diabetic patients

Age Interval years	Number of Patients	Ergothioneine mg.% corpuscles	Standard error of mean
2 - 39	14	14.2	2.25
40 - 49	14	12.9	1.52
50 - 59	15	17.5	2.70
60 - 69	20	12.3	2.70
70 - 89	13	8.2	0.76
0 - 59*	43	14.9	1.27
59 - 89*	33	10.7	1.69

*t value found = 1.15
t value at 5% level = 1.994

On comparing the age group 2-59 (43 patients) with the age group 60-89, (33 patients) the difference of the means was not found to be significant.

The state of diabetic control and the duration of diabetes, two factors which might have borne some relation to ergothioneine level in diabetics, were then examined.

TABLE 17

Blood ergothioneine levels in well controlled and poorly controlled diabetics

	Poorly Controlled	Well Controlled
Number of patients	47	35
Mean ergothioneine level mg.%	13.8	12.1
Standard error of the mean	1.46	1.18

t value found = 0.481
t value at 5% level = 1.990

Table 17 shows a comparison of the poorly controlled and the well controlled diabetics in the series. A history of the state of control was obtained in 82 of the 107 patients. A comparison showed that no significant difference existed in ergothioneine levels of well and poorly controlled diabetics. In 17 diabetics previously referred to who had levels higher than the highest normal value of 17.7 mg.%, 11 or 64.7% were severe diabetics or were poorly controlled. There were, however, 6 (35.3%)

patients with high values who were well controlled and often mild diabetics.

A comparison was made between diabetics who had a history of the disease for less than 10 years and those who had been diabetics for 10 years or longer. The history of duration of diabetes was obtained in 64 of the 107 patients.

TABLE 18

Blood ergothioneine levels in diabetics with disease duration of greater and less than 10 years

	Diabetics for less than 10 years	Diabetics for 10 years and longer
Number of patients	39	25
Mean ergothioneine level mg.%	12.2	14.3
Standard error of the mean	1.25	2.19

t value found = 0.506
t value at the 5% level = 2.000

There was no significant difference between the means of the diabetics with recent disease or those with long-standing diabetes, as shown in Table 18.

Two patients, admitted in diabetic coma, with blood glucose levels of over 400 mg.% and CO_2 combining power of 40 vol.%, were found to have normal ergothioneine values which did not change on control of their

diabetic state.

One patient in insulin shock with a blood glucose of 47 mg.% showed an increase of ergothioneine to 17.1 mg.% from a previous reading of 6.9 mg.% the same day, with a return to the lower level when next tested five days days later. Because of this observation samples of blood were taken from four patients being treated with sub-shock doses of insulin on a pyychiatric service. These were obtained at six-thirty in the morning and again about nine-thirty a.m., by which time the symptoms of hypoglycemia from the early morning insulin were maximal. There was no difference in the ergothioneine levels in any pair of specimens.

In eleven patients from whom samples had been obtained at various times during the same day, usually at 8 a.m., 11 a.m. and 2 p.m., there was no apparent relation to meals or to the time of day at which the specimens were taken.

A diabetic patient examined pre-operatively and post-operatively (hip-nailing) showed a post-operative rise to 16.1 mg.% of ergothioneine from a previous level of 9.4 mg.%, and still showed the higher level four days later. No other operative patients were examined.

The lowest value in diabetes - 2.0 mg.% - was found in a sixty-eight year female who was controlled

on 15 units of P.Z. insulin, and who was also suffering from hypertension and senile psychosis. Another low value of 4.2 mg.% was observed in a patient who had a fasting glucose of 250 mg.% and who was also being treated for cystitis and mild congestive heart failure.

Ergothioneine estimations were made on a well controlled diabetic student at monthly intervals. The small fluctuations seen in Figure 3 cannot be explained by any change in his diet, which is necessarily more restricted than that of a normal person, nor by any change in diabetic control which has been excellent over this period. Similar small fluctuations can be seen in ergothioneine levels of 4 normal subjects taken over comparable periods of time.

Non-protein nitrogen, uric acid, and blood cholesterol values in 25, 34 and 54 patients respectively, showed no correlation with the ergothioneine content of the same blood.

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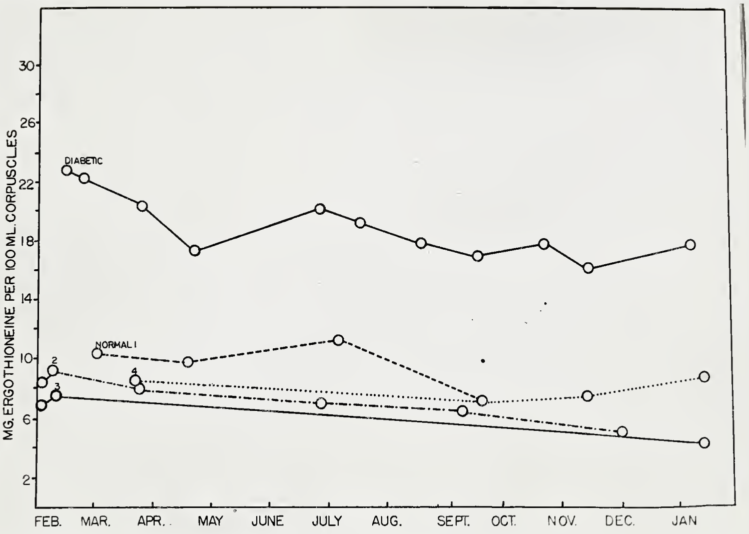

Figure 3 Periodic blood ergothioneine levels of one diabetic student and four normal subjects.

TUBERCULOSIS

Blood ergothioneine levels were determined in 65 Indian and 4 Eskimo patients, all with some type of tuberculosis. The mean ergothioneine value for these 69 patients was 18.0 mg.%, a level well above the normal mean of 9.6 mg.% (Table 19). This series was divided into smaller groups according to type of tuberculosis and the clinical degree of infection. Although it may not be a significant finding, it is interesting to note the gradual decrease in mean ergothioneine value which corresponds to the increase in severity of the tuberculosis. For example, two patients who subsequently died had levels of 7.6 and 19.0 mg.%, and three others with far advanced tuberculosis showed levels of 6.7, 6.9 and 11.5 mg.%. As shown in Table 19 the mean ergothioneine value for far advanced tuberculosis was approximately 5 mg.% lower than that for controlled or minimal tuberculosis.

Single cases each of renal tuberculosis and tuberculoma exhibited extraordinarily high values.

In the course of the studies on tuberculosis in Indians,we made estimations on three patients* who were admitted as tuberculosis suspects, and who sub-

*Not included in Table 19.

TABLE 19

Blood ergothioneine levels of 65 Indian and 4 Eskimo patients with Tuberculosis

	Number of patients	Mean Age (years)	Mean Ergothioneine mg.% corpuscles
Normal Subjects	94	29.5	9.6
All patients with tuberculosis	69	18.0	18.0*
Controlled or minimal pulmonary tuberculosis	24	15.6	19.1
Moderately advanced pulmonary tuberculosis	18	20.0	16.8
Far advanced pulmonary tuberculosis	15	23.8	14.2
Tuberculosis of bone	10	12.8	14.8
Tuberculoma	1	8	48.2
Renal tuberculosis	1	15	52.3

Range 4.3 - 53.0 mg.%
*Standard error of the mean 1.5
*t value found = 3.674
t value at 1% level = 2.609

sequently proved to be negative. We also determined the ergothioneine level of one patient admitted for treatment of a congenital dislocation of the hip. The surprising findings were ergothioneine values of 14.5, 23.6, 22.1 and 42.6 mg.% respectively. The first three cases all suffered from some other lung disease, but the three year old boy with the congenital hip dislocation was apparently otherwise well.

We felt that peculiarities in diet might account for differences in ergothioneine levels in these races, but on inquiry we found that many cases showing high ergothioneine values had been on routine hospital diets for as long as three years. We also noted that new admissions frequently showed values close to normal limits.

We then attempted to make a series of estimations on blood from white patients with tuberculosis. Although approximately 50 samples were obtained, few ergothioneine values were determined because of the high incidence of interference. These estimations were made in part prior to the development of the washing-technique for overcoming interference; many other samples had too little blood to permit washing. As a result only 9 estimations were completed. The results are given in Table 20. The range of ergothioneine

values was 6.8 - 20.0 mg.%. The difference in means (normal white and tuberculous white patients) was found to be statistically significant.

TABLE 20

A comparison of the mean blood ergothioneine value of 9 tuberculous white patients with the mean value of 94 normal subjects

	Normal Subjects	Tuberculous Patients
Number of patients	94	9
Mean ergothioneine level mg.%	9.6	12.8
Standard error of the mean	0.3	1.5

t value found=3.190
t value at the 1% level = 2.632

. VARIOUS OTHER DISEASES

Small numbers of patients with various other diseases were investigated when the opportunity arose. To facilitate analysis, some of these were grouped under broader classifications. An attempt was made to do this only with closely related conditions. These are presented in ascending order of the mean ergothioneine values, together with their ranges, in Table 21. The significant clinical and laboratory findings are presented for each individual patient in tables to be found in the addendum. In some cases complete histories were not available from the hospital records, but all available abnormal or significant findings are given.

In reviewing the 23 conditions presented in Table 21 we felt that the means of the ergothioneine values in the diseases numbered from 16 to 23 were sufficiently high to justify a statistical comparison with the normal mean. The standard deviation, standard error of the mean, t value calculated and t value at the 1% and 5% level of significance are shown in Table 22. There is a highly significant increase in mean ergothioneine values found in diseases numbered 18 - 23. It was interesting to find that the mean of the group

TABLE 21

Number, Range and Mean ergothioneine levels of various diseases

	Number of cases	Range of values mg.%	Mean ergothioneine level mg.%
*1 Hypothyroidism	5	3.5 - 13.4	6.7
2 Hernia	4	3.6 - 10.9	7.7
3 Pregnancy	4	4.3 - 13.0	7.8
4 Arthritis, rheumatoid and osteoarthritis	9	1.5 - 15.6	8.3
5 Rheumatic fever and rheumatic heart disease	5	2.9 - 14.6	8.5
6 Hyperthyroidism	9	5.0 - 13.0	8.7
7 Leukemia and multiple myeloma	4	8.8 - 10.4	9.4
8 Appendicitis	10	3.9 - 17.9	9.5
9 Infections other than respiratory	12	4.1 - 15.5	9.6
10 Abortions	9	5.0 - 13.5	9.8
11 Chest diseases	23	2.6 - 20.3	9.9
12 Anemias	6	2.7 - 18.8	10.2
13 Carcinoma	17	2.6 - 33.5	10.7
14 Psychoneuroses and psychoses	8	3.1 - 16.2	10.8
15 Peptic ulcer	6	3.0 - 19.4	10.8
16 Gall bladder disease	7	4.1 - 23.8	12.4
17 Coronary thrombosis, and insufficiency, and hypertensive cardiovascular disease	17	3.3 - 25.5	12.4

*Numbers corresponding to classification in addendum.

Table 21 continued.

18	Diabetes mellitus with associated diseases	8	7.4 - 32.1	12.7
19	Fractures	6	7.9 - 19.1	13.2
20	Nephritis, nephrosis, and urinary obstruction	12	6.7 - 31.3	13.4
21	Infections, upper respiratory	9	7.6 - 26.8	13.5
22	Gynaecological surgical conditions	5	8.2 - 18.4	13.6
23	Dermatitis	7	6.0 - 26.3	14.8

"Diabetes mellitus with associated diseases" was identical with the value found for the mean in the larger number of diabetics reported earlier in this paper who were suffering from no other major disease.

All cases were then listed in which the ergothioneine value exceeded the highest normal value of 17.7 mg.%. In Table 23 the history and laboratory findings of each patient are given in the hope of finding some common factor. Such as factor is not apparent. The table includes five other categories as well as the eight (16-23) listed in Table 22. Two of the five patients had been given transfusions prior to the ergothioneine determination; one of the other three was suffering from some kidney failure. The only high value from a miscellaneous group of diseases (Addendum, A.) was found in blood from a young woman with chronic pelvic inflammatory disease.

Blood ergothioneine levels were determined on two patients receiving cortisone. The first was a case of leukemia who was moribund when the blood sample was obtained, four days after starting the drug. There was no change from the initial blood ergothioneine level of 9.4 mg.%. The second was a case of lupus erythematosis in whom the initial ergothioneine level was 11.3 mg.% (the test being performed on washed cells because of interference.). On March 14 cortisone therapy was started.

TABLE 22

A comparison of the mean blood ergothioneine values found in 8 diseases with mean value in normal subjects

	Disease	Number of patients	Ergothioneine mg.% corpuscles	Standard deviation	Standard error of mean	t value* found
16	Gall bladder disease.	7	12.4	6.2	2.3	2.147
17	Coronary thrombosis, insufficiency and hypertensive cardio-vascular disease.	17	12.4	7.5	1.8	2.613
18	Diabetes mellitus with associated diseases.	8	12.7	9.1	3.2	3.450
19	Fractures.	6	13.2	4.4	1.8	2.693
20	Nephritis, nephrosis and urinary obstruction.	12	13.4	6.7	1.9	3.405
21	Infections, upper respiratory.	9	13.5	6.5	2.2	3.186
22	Gynecological surgical conditions.	5	13.6	4.0	1.8	2.750
23	Dermatitis.	7	14.8	7.2	2.7	3.788
	Normal Subjects.	94	9.6	3.1	0.3	-

*t value at 5% level = 1.987; at 1% level = 2.632

TABLE 23

Findings in cases with blood ergothioneine values greater than the highest value in normal persons (17.7 mg.%)

		Ergothioneine mg.%	% cases from each category*
8.	Appendicitis		
	Acute, ruptured. Age 47	17.9	10
11.	Chest diseases		
	Bronchiectasis, Asthma (48 years) Consolidation of lower lobe. Age 65.	18.3	8.7
	Atypical virus pneumonis. Bronchiectasis. Age 63	20.3	
12.	Anaemias		
	Hypochromic. Menorrhagia. Haemoglobin 8.1 gm.% Transfusions.	18.8	16.6
13.	Carcinoma		
	Stomach (i year) Transfusion 1 day previous. Haemoglobin 3.6 gm.%. Age 79	25.9	11.7
	Prostate with metastases. (1 year) Pleural effusion. Age 70	33.5	
15.	Peptic ulcer		
	Gastric. Age 41	19.4	16.6
16.	Gall bladder disease		
	Cholelithiasis, Acute cholecystitis, (no jaundice) Pyuria Albuminuria, age 61	23.8	14.3
17.	Coronary thrombosis, and insufficiency; hypertensive cardiovascular disease		
	Coronary insufficiency. Obesity. Age 54	23.4	23.5

Table 23 continued.

	Coronary thrombosis. Sedimentation rate 25 mm. per hour. Age 53	24.9	
	Coronary insufficiency. B.P. 200/130. Age 54	25.5	
	Coronary thrombosis	24.2	
18.	Diabetes mellitus with associated diseases		
	Diabetes, Lobar pneumonia (5 days) Massive collapse of one lower lobe, with effusion. Age 76	32.1	28.6
	Diabetes. Nephritis and pyelitis (5 days) N.P.N. 35 mg.% B.P. 180/90. Age 56	24.5	
19.	Fractures		
	Fractured os calcis. Age 43	19.1	16.6
20.	Nephritis, nephrosis, and urinary obstruction		
	Nephrosis (1 year) Serum protein 4.4 gm.%, Albumin 2.3 gm.% Cholesterol 1000 mg.%. Age 6	19.4	16.6
	Toxic nephrosis. Septic abortion. 2 months pregnant. N.P.N. 235 mg.%	31.3	
21.	Infections, upper respiratory		
	Acute pharyngitis and tonsillitis. Age 22	21.0	22.2
	Tonsillitis. Age 29	26.8	
22.	Gynecological surgical conditions		
	Prolapse. Age 35	18.4	20.0

Table 23 continued.

23.	Dermatitis, eczema and urticaria		
	Urticaria. Age 37	21.8	28.6
	Stasis dermatitis. B.P. 160/70. Age 81	26.3	
A.	Miscellaneous		
	Low back strain, Old pelvic inflammatory disease with spotting (blood) and discharge. Age 32	30.5	4.3

*Percentage of cases, from each disease classification, which have values greater than 17.7 mg.%.

Blood ergothioneine levels on March 18, March 22 and March 31 were 7.6, 8.2 and 8.8 mg.% respectively. Cortisone therapy had no apparent effect on blood ergothioneine levels in these two patients.

We took the opportunity of making simultaneous ergothioneine estimations on peripheral blood and bone marrow in a patient suffering from aleukemic leukemia. An ergothioneine value of 10.4 mg.% was found in the peripheral blood, and a higher level of 14.9 mg.% in the bone marrow. This difference in values was suggestive of increased concentration of ergothioneine in young cells. This possibility was further supported by the finding that ergothioneine values increased, after 3 to 4 days, in rats which were subjected to repeated bleeding during that period of time (33).

EXPERIMENTAL

The purpose of this investigation was to determine whether there was any variation in blood ergothioneine in human disease states, and whether there was any significant difference between these values and those found in normal human blood. The work so far presented shows that ergothioneine values fall within a moderately narrow range in normal subjects. It also demonstrates that a wide range of values can be expected in nine disease categories. From our examination of these findings there does not appear to be any factor common to these diseases. Certain minor experimental problems did occur to us, however, and these are described below.

Ergothioneine in Alloxan Diabetes

A number of reports have been published on the protection of experimental animals from alloxan diabetes by the prior injection of glutathione, cysteine and related compounds (34, 35, 36, 37). In conjunction with another member of this department (Mr. D.W. Gee), who is reporting this work in full, we conducted a similar experiment on 4 rats, using ergothioneine as a "protecting" substance. All four control rats developed diabetes following the intravenous injection of alloxan (40 mg. per Kg. body weight). Three of the four rats

which were given ergothioneine intravenously (0.09 millimols)* 1 - 3 minutes prior to the injection of alloxan, failed to develop diabetes. This result appeared to be significant because the reports in the literature suggested that 0.75 - 7.00 millimols*of glutathione, and equivalent amounts of other effective compounds, are needed to protect the rat from alloxan diabetes. Other workers (34) who used the same amount of intravenous alloxan as we did, found that the smaller amount protected 60 - 100% of the rats from developing diabetes. It can be seen that the amount of ergothioneine that we injected when calculated in millimols, was equivalent to only 1/8 to 1/10 of the amounts of other sulfhydryl containing substances which have been found necessary to give comparable protection.

The Effect of Blood Loss on Ergothioneine Values

Work done by another member of this department (Miss M.S. Fraser) on ergothioneine levels in rats suggested that an increase in ergothioneine values occurred when rats were bled. For this reason estimations were made on students who donated blood to the Red Cross Blood Donor Clinic. These estimations carried out before the donation, and at 1 and 2 weeks after the donation. The results are tabulated in Table 24.

*Per Kg. body weight.

TABLE 24

Blood ergothioneine levels of seven normal subjects before and after blood donations.*

Student	Before donation Ergothioneine mg.%	1 week after donation Ergothioneine mg.%	2 weeks after donation Ergothioneine mg.%
A	6.9	3.9	7.9
B	4.1	7.9	5.7
C	8.0	6.4	9.3
D	7.3	6.3	7.7
E	5.9	6.3	6.7
F	8.5	6.5	10.3
G	6.2	7.6	8.4

*Approximately 350 ml.

At the end of one week ergothioneine values showed both increases and decreases from the initial levels. By the end of the second week the ergothioneine levels of all subjects were higher than their respective initial values, even though in some cases the difference was small.

Perhaps we may be permitted to speculate upon these findings. Rats used in this experiment were kept on a diet of constant quality, and for that reason the available amount of ergothioneine, or any precursor of that substance, was presumably constant. Why, then,

should an increase in haematopoietic activity have caused an increase in concentration of corpuscular ergothioneine? There is no reason to expect that bleeding per se should cause an increase in the amount of ergothioneine incorporated into each cell. Imagine then, that every red blood cell begins its life with a proportionately higher and completes it with a relatively lower concentration of ergothioneine. The ergothioneine value per cent corpuscles would then depend on three factors:

1. the availability to the organism of ergothioneine or its precursor.
2. the size of the erythrocyte.
3. the proportion of young to mature erythrocytes in the blood.

The following findings suggest that this aspect of the problem should be investigated.

1. Blood ergothioneine levels of rats are affected by changes in the diet (33).
2. Repeated bleeding of rats, and to a lesser extent blood loss in normal human subjects appear to be associated with a rise in ergothioneine values.
3. In the one case investigated, a higher ergothioneine level was found in human bone marrow than in the corresponding peripheral blood.

Fate of Ergothioneine Injected Intravenously Into Rats

Ten mg. of ergothioneine was injected into the tail vein of a normal rat. One hour later the blood ergothioneine level was exactly equal to the pre-injection level. We had noted high ergothioneine levels in some patients suffering from kidney failure, and for this reason we wished to learn whether ergothioneine might be excreted by the kidneys. No satisfactory test was available for the quantitative measurement of ergothioneine in the urine. Working in conjunction with another member of this department (Miss M.S. Fraser) who is reporting this work in full, we performed bilateral nephrectomies on two rats. Ten mg. of ergothioneine was then injected into the tail veins of these nephrectomized rats. One hour later the whole blood of each showed an increase in ergothioneine value, but in both cases the increase was shown to be due to the presence of ergothioneine in the plasma, from which it is normally absent. The ergothioneine value of the corpuscles remained constant. We concluded that when ergothioneine is injected into a normal rat the kidneys play a part in excreting the excess. We did not attempt a quantitative explanation for the disposition of the injected ergothioneine, but it was also apparent from the degree of increase in blood ergothioneine after

injection that much of the ergothioneine had been removed from the circulation. In these two rats it could not have been removed by the kidneys. One of the rats was given a second injection of 10 mg. of ergothioneine intramuscularly. After the second injection we found a further increase in blood ergothioneine, and all of this increase appeared in the plasma. Once more we were unable to account for all the material injected. From these experiments three conclusions were reached:

1. In the normal rat injected ergothioneine is quickly removed from the circulating plasma, and the kidneys appear essential for at least part of this function.
2. Ergothioneine is removed to a considerable degree from the plasma of rats in which urinary excretion is prevented; or it exists in some other form than ergothioneine.
3. There is no appreciable transfer of ergothioneine from plasma to cells in vivo.

Plasma-erythrocyte Transference of Ergothioneine in vitro

Two aliquots of a blood sample were taken and an unweighed amount of ergothioneine (prepared by Miss M.S. Fraser) was added to one. Wach aliquot was well mixed and allowed to stand for one hour at room temperature. The plasma and cells were then separated, and the cells of each aliquot washed 4 times with saline to remove any plasma.* The cells of the aliquot to which the ergothioneine had been added gave an ergothioneine value of

9.8 mg.% and the cells of the aliquot to which no ergothioneine had been added gave a value of 10.1 mg.%. The plasma ergothioneine value of the aliquot with added ergothioneine was 5.7 mg.%. The plasma of the control aliquot gave the usual "ergothioneine-equivalent" value of 1.2 mg.% (no ergothioneine colour).

A second sample of normal fresh blood was also divided into two lots and ergothioneine added to the second aliquot. Both aliquots were mixed thoroughly and allowed to stand for one hour. The increase found in the second aliquot was equal to 2.3 mg.% ergothioneine. The plasma of the second aliquot gave a value of 5.2 mg.%. When the plasma percentage (100 - haematocrit) of the second aliquot was taken into consideration, the ergothioneine value contributed to the whole blood ergothioneine reading of the second aliquot was 2.1 mg.%. This is close to the total increase determined for this aliquot (2.3 mg.%). The plasma, therefore appeared to contain all the added ergothioneine.

This evidence suggests that the red cell membrane is impermeable to ergothioneine, or at least that there is no free plasma-erythrocyte diffusion. The incorporation of ergothioneine into the red cell during its

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*No appreciable amount of ergothioneine was found in the saline washings.

initial stages of development would explain the rather slow appearance of changes in ergothioneine values in experimental animals.

CONCLUSION

There are many aspects of this problem which should be explored. Comparatively few biochemical or physiological studies have been made. Our work shows that these are indicated. In all normal subjects who were investigated there was a range of only 14 mg.%, and a standard deviation of 3.1 mg.%. The abnormal findings in various diseases emphasize the yet unanswered question of function. It seems unlikely that there would be no role assigned to a constituent of such a vital structure as the red blood cell, especially since ergothioneine has been shown to possess a definite oxidation-reduction potential, (38).

SUMMARY

1. The Hunter diazo test was used for the determination of blood ergothioneine values in 401 reported patients and 94 normal subjects.
2. The calculation of the amount of ergothioneine per cent corpuscles was modified by taking into consideration the additional value contributed by plasma.
3. The problem of the development of an interfering colour in the test was investigated and methods suggested for avoiding and removing this effect.
4. A range and mean ergothioneine value were established for normal subjects.
5. Blood from 107 diabetic patients was compared for mean ergothioneine content with blood from 94 normal subjects. A highly significant increase was demonstrated in the mean value for diabetics. No relationship was established between the blood ergothioneine level and any one of the following factors: diabetic state of control, duration of diabetes, age, sex, blood cholesterol, uric acid, or non-protein nitrogen.
6. Blood ergothioneine levels*of both white and native (Indian and Eskimo) patients were found to be significantly higher than the mean ergothioneine

*Mean

of normal white subjects. No ergothioneine estimations were made on a normal native population. Four native patients with diseases other than tuberculosis had high ergothioneine levels.

7. Blood ergothioneine estimations were made on 294 other patients with various diseases. Increases in the ergothioneine mean which were statistically significant were found in gall bladder disease, coronary disease and hypertensive cardiovascular disease, diabetes with associated disease, fractures, upper respiratory infections, gynecological operative conditions, nephritis, nephrosis and urinary obstruction, and dermatitis.
8. No absorption of ergothioneine by the corpuscles from plasma could be shown either in vivo or in vitro.
9. Ergothioneine,injected into rats which had been subjected to bilateral nephrectomies, was found only in the plasma fraction of the blood.
10. The desirability of further studies on the physiology and pharmacology of this blood constituent is stressed.

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ADDENDUM

1. Hypothyroidism		Age	Ergothioneine mg.%
Hypothyroidism.	B.M.R. minus 15	23	3.5 approx.
Hypothyroidism.	B.M.R. minus 14	22	3.9
Myxoedema.	Blood Cholesterol 284 mg.% B.M.R. minus 42	17	4.6
Hypothyroidism?	Psychoneuroses.	40	8.3
Hypothyroidism.	Old Pott's disease of spine, inactive. Pharyngitis. B.M.R. minus 14.	53	13.4
Ergothioneine range for cases of hypothyroidism.		3.5 - 13.4 mg.%	
Ergothioneine mean for cases of hypothyroidism.		6.7 mg.%	

2. Hernias		Age	Ergothioneine mg.%
Hernia.	(2 days post-op) B.P. 180/100	64	6.0
Hernia.		62	10.3
Hernia		18	10.9
Hydrocoele.	Polycythemia vera diagnosed 3 years previously	71	3.6
Ergothioneine range in cases of hernia and hydrocoele.		3.6 - 10.9 mg.%	
Ergothioneine mean in cases of hernia and hydrocoele.		7.7 mg.%	

3. Pregnancy

	Age	Ergothioneine mg.%
Full term. Delivered 10 days previously.	40	4.3
Toxaemia of pregnancy. 8 months.	19	5.8
Ectopic pregnancy. 5 weeks.	35	7.9
Pre-eclampsia. Full term.	24	13.0
Ergothioneine range for all cases of pregnancy.	4.3 - 13.0 mg.%	
Ergothioneine mean for all cases of pregnancy.	7.8 mg.%	

4. Arthritis

Rheumatoid (9 years). Coronary insufficiency.	60	5.4
Rheumatoid. B.P. 180/105	62	7.5 approx.
Early rheumatoid. Prostatism. B.P. 180/88	75	8.3
Rheumatoid. (3 years) 3 months pregnant.	--	12.4
Osteoarthritis. Coronary sclerosis. B.P. 160/100	76	1.5
Arthritic back pain (5 years)	35	7.8
Osteoarthritis and anxiety state. B.M.R. plus 28	49	8.0 approx.

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	Age		Ergothioneine mg.%
Osteoarthritis. Dorsal kyphosis.	56		8.0 approx.
Osteoarthritis of spine. B.P. 152/104	79		15.6
Ergothioneine range	1.5 - 15.6 mg.%		
Ergothioneine mean	8.3 mg.%		

5. Rheumatic Fever and Rheumatic Heart Disease

	Age		Ergothioneine mg.%
Rheumatic arthritis. Gastric diverticulum. B.P. 160/100	72		2.9
Acute rheumatic fever. (2 weeks)	8	Feb.1 Feb.2	7.7 7.9
Rheumatic heart disease. Varicose veins.	71		7.9
Acute rheumatic fever. Obese.	14		9.2
Rheumatic heart disease, active.	24		14.6
Ergothioneine range	2.9 - 14.6 mg.%		
Ergothioneine mean	8.5 mg.%		

6. Hyperthyroidism

	Age	Ergothioneine mg.%
Adenomatous goitre. Toxic pregnancy 6 months	42	5.6
Adenoma of the thyroid. B.M.R. minus 1	24	7.7
Hyperthyroidism. (for 3 months)	26	5.0

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	Age	Ergothioneine mg.%	
Hyperthyroidism. B.M.R. plus 7-27	39	Sept.3 Sept.9	8.1 4.4
Hyperthyroidism with exophthalmos.	22		8.0 approx.
Hyperthyroidism. Epidermoid carcinoma of lip removed 4 months previous.	34	Sept.2 Sept.2 Sept.19	9.3 9.6 7.8
Hyperthyroidism. Thyroidectomy 7 days previous. Cerebral embolism hepatic cirrhosis, portal.	57		11.1
Hyperthyroidism. Thyroidectomy Feb.2. Toxic goitre. B.M.R. plus 69 Urea clearance 36% Albuminuria B.P. 250/140	54	Jan.24 Mar.3	12.9 12.4
Hyperthyroidism? Anxiety state. (for 6 months)	29		13.0
Ergothioneine range.	5.0 - 13.0 mg.%		
Ergothioneine mean.	8.7 mg.%		

7. Leukemia and Multiple Myeloma

Multiple myeloma. Serum calcium 14.1 Bence Jones proteinuria	58		8.8
Aleukemic leukemia. Lobar pneumonia. Pericarditis.	47		9.5

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	Age	Ergothioneine mg.%
Myelogenous leukemia.	--	Feb.17 8.4 Feb.20 9.4 Feb.25 9.5
Aleukemia leukemia.	23	10.4
Ergothioneine range	8.8 - 10.4 mg.%	
Ergothioneine mean	9.4 mg.%	

8. Appendicitis

	Age	Ergothioneine mg.%
Appendicitis. Sub-acute.	8	3.9
Appendicitis. Chronic. (1 day post-op)	23	4.2
Appendicitis. Chronic.	10	7.4
Appendicitis. Gangrenous. (1 day post-op)	18	6.6
Normal appendix.	8	9.2
Appendicitis.	--	9.4
Appendicitis. Acute ulcerative.	23	10.3
Appendicitis. Chronic recurrent.	39	10.7
Appendicitis. Ruptured.	47	17.9
Appendicitis. Pin-worm infestation. B.P. 160/90	17	15.7

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	Age	Ergothioneine mg.%
Ergothioneine range	3.9 - 17.9 mg.%	
Ergothioneine mean	9.5 mg.%	
9. Infections, Other than Upper Respiratory		
Throbophlebitis.	28	4.2 4.0
Salmonella infection.	20	4.8
Diarrhoea of undetermined cause.	58	7.1
Cellulitis of the thigh.	--	8.7
Rectal abscess.	--	9.3
Infected hand.	--	9.6
Ischio-rectal abscess.	33	9.8
Infected knee.	--	10.1
Cellulitis of hand with lymphadenitis.	--	11.3
Chronic patellar bursitis and hypertension.	65	12.1
Pelvic abscess.	46	13.0
Ergothioneine range	4.1 - 15.5 mg.%	
Ergothioneine mean	12.9 mg.%	

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10. Abortions and Threatened Abortions

	Age	Ergothioneine mg.%
Pregnant. Threatened abortion. Syphilis?	32	5.0
Threatened. 4-5 months pregnant.	30	6.0
Threatened. 3 months pregnant.	35	6.4
Threatened.	32	9.2
Hyperemesis gravidarum. 6 weeks pregnant.	27	9.7
Threatened. 2 months pregnant.	21	12.3
Threatened. 10 weeks. 3 weeks vomiting.	24	13.0
Threatened. 5 transfusions.	30	13.3
Threatened. 3 months pregnant.	25	13.5
Ergothioneine range	5.0 - 13.5 mg.%	
Ergothioneine mean	9.8 mg.%	

11. Chest Diseases

Diaphragmatic pleurisy (3 days)	30	10.5
Bronchiectasis. Asthma. (48 years) Consolidation of lower lobe.	65	18.3

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	Age	Ergothioneine mg.%
Bronchopneumonia.	40	6.6
Bronchopneumonia. Gout. Uric acid 4.2 mg.%	62	7.6
Bronchopneumonia. (8 days) Asthmatic.	57	11.2
Bronchopneumonia. Alcoholism.	36	12.4
Bronchopneumonia. Pleurisy.	53	17.4
Atypical virus pneumonia. Atelectasis. N.P.N. 58 mg.% Serum Protein 6.9 gm.%, Albumin 2.92 gm.%	58	2.6
Atypical virus pneumonia. 5 months pregnant. Rheumatoid arthritis. Gold therapy.	34	3.6
Atypical virus pneumonia.	23	7.0 approx.
Atypical virus pneumonia.	--	8.5
Atypical virus pneumonia.	19	8.6
Atypical virus pneumonia.	--	14.1
Atypical virus pneumonia. Bronchiectasis.	63	20.3
Pneumonia (not further identified)	--	7.0 approx.
Pneumonia.	40	8.7
Pneumonia.	--	10.0 approx.
Lobar pneumonia. Chronic cough 20 years.	35	4.3

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	Age	Ergothioneine mg.%
Lobar pneumonia. Chronic cough 20 years.	32	14.1
Chronic bronchitis. Radicle mastectomy 2 years previously. B.P. 180/115	65	7.6
Asthma, acute. Pyuria.	47	8.0
Asthma. Localized infiltration of lung.	52	8.6
Asthma. Emphysema. Cataracts.	46	11.7
Ergothioneine range	2.6 - 20.3 mg.%	
Ergothioneine mean	9.9 mg.%	

12. Anaemias

	Age	Ergothioneine mg.%
Pernicious. Thyroidectomy 9 years ago. Rheumatic heart disease. B.M.R. plus 60. B.P. 170/60 Red blood count 1.65 million.	45	2.7
Pernicious. (7-8 years) Gastroenterostomy (18 years ago) Haemoglobin 6.3 gm.%.	48	5.4
Hypochromic anaemia. Thyroidectomy (7 years ago) Blood Cholesterol 254 mg.%, Albuminuria.	43	11.7
Hypoplastic anaemia. Transfusions.	62	17.1

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	Age	Ergothioneine mg.% Corpuscles	
Hypochromic anaemia. Menorrhagia. Haemoglobin 8.1 gm.% Transfusions.	30	Jan.27 Feb.21	18.4 19.1
Anaemia.	--		5.7
Ergothioneine range	2.7 - 18.8 mg.%		
Ergothioneine mean	10.2 mg.%		
13. Carcinoma			
Colon.	76		2.6
Osteochondroma, tibia.	8		3.3 approx.
Prostate (1 year) N.P.N. 145 mg.%. B.P. 185/100	62		4.6
Ewing's sarcoma, humerus, with metatases to chest.	9		5.0 approx.
Rectum with metastases. Colostomy for 9 months.	61		5.9
Adenocarcinoma of unknown source. (lyear) Haemoglobin 9.6 gm. Transfusion 3 days previous. 20% nucleated R.B.C. in smear	36	Sept. Mar.	7.9 5.4
Stomach (3 months)	77		7.2
Prostate (2 years) with matastases.	53		7.3

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	Age	Ergothioneine mg.% Corpuscles
Prostate. Orchidectomy 2 years ago.	75	8.0
Prostate with metastases. N.P.N. 70-90 mg.% B.P. 170/80	72	8.0 approx.
Prostate with metastases. Osteoarthritis	--	9.1
Breast	23	9.7
Uterus (2 years)	50	12.3
Pancreas (6 months) Indirect serum bilirubin 1.7 mg.%	40	14.7
Prostate with metastases. Osteoarthritis. N.P.N. 25 mg.%	78	18.2
Prostate with metastases (1 year) Pleural effusion.	70	33.5
Ergothioneine range	2.6 - 33.5 mg.%	
Ergothioneine mean	10.7 mg.%	

14. Psychoneurotic

Schizophrenic	37	3.1
Tension state.	44	4.4
Anxiety state. B.M.R. minus 12	35	9.6
Psychoneurotic.	38	9.6
Psychoneurotic. Very obese. B.P. 180/90	63	14.2

continued on next page

	Age		Ergothioneine mg.%
Psychoneurotic. Xanthoma of the eyelid.	38		14.5
Hysterical.	26		14.8
Hysterical paralysis. Serum protein 5.92 gm.% albumin 3.84 gm.%.	41		16.2
Ergothioneine range	3.1 - 16.2 mg.%		
Ergothioneine mean	10.8 mg.%		

15. Peptic Ulcer

	Age		Ergothioneine mg.%
Perforated duodenal ulcer. Pleural effusion.	38		3.0
Duodenal ulcer. Perforated 1 day after ergothioneine test	49		4.4
Post-gastectomy rupture of duodenal stump, peritonitis, acute pyelonephritis, hepatitis, N.P.N. 182 mg.%.	33		9.2
Duodenal ulcer.	57		12.8
Gastric ulcer.	41		19.4
Haemorrhaging ulcer. (8 days from January 16.)	32	Jan. 24 Jan. 26 Jan. 27	12.7 17.4 17.6
Ergothioneine range	3.0 - 19.4 mg.%		
Ergothioneine mean	10.8 mg.%		

16. Gall Bladder Disease

	Age	Ergothioneine
Cholecystitis	--	4.1
Cholelithiasis, Chronic cholecystitis. (no jaundice)	42	7.9
Cholecystitis	44	9.7
Cholelithiasis, chronic cholecystitis. (no jaundice) B.P. 220/110	68	12.9
Cholecystitis, pulmonary embolus 7 days post-operative, B.P. 170/110 Pleural effusion. Ergothioneine taken 11 days post-op.	49	13.8
Cholelithiasis (no jaundice)	38	14.6
Cholelithiasis, acute cholecystitis, (no jaundice) pyuria, albuminuria.	61	23.8
Ergothioneine range	4.1 - 23.8 mg.%	
Ergothioneine mean	12.4 mg.%	

17. Coronary Thrombosis, Insufficiency, and Lypertensive Cardiovascular Disease

	Age	Ergothioneine
Coronary Thrombosis. (2 weeks before)	43	7.2
Coronary Thrombosis (4 days before) Sedimentation rate 25 mm. per hour.	80	7.1

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	Age		Ergothioneine mg.%
Coronary thrombosis. Pneumonitis. Syphilis?. Urea clearance 61%. Obese.	53		9.6
Coronary insufficiency. Arteriosclerotic heart disease	63		9.9
Coronary thrombosis. (2 and 7 days before)	55	2 days 7 days	13.2 14.4
Coronary insufficiency. Obesity	54		23.4
Coronary thrombosis	--		24.2
Coronary thrombosis. Sedimentation rate 25 mm. per hour.	53		24.9
Coronary insufficiency. B.P. 200/130	54		25.5
Hypertensive cardiovascular disease. B.P. 205/130	49		3.3
Hypertensive cardiovascular disease. Inactive pulmonary tuberculosis. B.P. 200/110.	77		5.1
Hypertensive cardiovascular disease. Congestive heart failure. B.P. 220/130.	50		5.3
Hypertensive cardiovascular disease. A-V-block. N.P.N. 69 mg.%. B.P. 205/75.	74		7.1
Hypertensive encephalopathy. 3 days before. B.P. 175/85	65		8.2

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	Age	Ergothioneine mg.%
Hypertensive cardiovascular disease. Congestive heart failure. B.P. 210/110.	83	9.6
Hypertensive cardiovascular disease. B.P. 230/130	85	12.5
Hypertensive cardiovascular disease. Pulmonary fibrosis. B.P. 160/100.	88	14.0
Ergothioneine range	3.3 - 25.5 mg.%	
Ergothioneine mean	12.4 mg.%	

18. Diabetes Associated With Other Diseases

	Age	Ergothioneine mg.%
Diabetes. Fractured pubis. (1 month previous)	55	7.4
Diabetes. Pulmonary tuberculosis.	--	Jan.30 6.6 Feb.20 8.4 Feb.22 8.4
Diabetes. Cellulitis of the thigh (for 5 days)	29	8.5
Diabetes. Psoriasis. B.P. 160/100	52	8.9
Diabetes. Hypertensive cardiovascular disease. B.P. 210/110. Carcinoma of the pancreas. N.P.N. 49 mg.% Alkaline phosphatase 86 K-A units. Pyuria. Jaundiced (markedly)	76	32.1

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	Age	Ergothioneine mg.%
Diabetes. Hyperthyroidism. B.M.R. plus 32.	--	15.6
Diabetes. Lobar pneumonia. (for 5 days) Massive collapse of one lower lobe, with effusion.	76	32.1
Diabetes. Nephritis and pyelitis (for 5 days) N.P.N. 35 mg.%. B.P. 180/90.	56	24.5
Ergothioneine range	7.4 - 32.1 mg.%	
Ergothioneine mean	12.7 mg.%	

19. Fractures

	Age	Ergothioneine mg.%
Ankle.	--	7.6
Tibia. Cataract.	64	9.7
Metacarpal. 13 days before determination.	21	11.2
Old ununited fracture tibia. No callus.	34	15.3
Fractured pubis. Lacerated femoral vein. 10 days before determination	31	16.4
Os calcis.	43	19.1
Ergothioneine range	7.6 - 19.1 mg.%	
Ergothioneine mean	13.2 mg.%	

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20. Nephritis, Nephrosis and Urinary Obstruction

	Age	Ergothioneine mg.%
Acute glomerular nephritis. N.P.N. 46 mg.%	15	11.3
Pyelo-nephritis. Agenesis right kidney. N.P.N. 92 mg.% B.P. 280/184	25	11.6
Acute glomerular nephritis. N.P.N. 42 mg.%. B.P. 204/122	34	15.9
Adenoma prostate (1 year).	63	6.7
Adenoma prostate. Pulmonary embolus. (10 days previous) Diabetic?	69	8.4
Adenoma prostate. N.P.N. 75	--	8.3
Adenoma prostate. N.P.N. 120	--	9.0
Acute urinary retention. Transurethral resection 3 years ago	67	12.4
Adenoma prostate. B.P. 157/70	79	15.6
Nephrosis. (1 year) Serum Protein 4.4 gm.%, Albumin 2.3 gm.%, Cholesterol 1000 mg.%.	6	19.4
Toxic nephrosis. Septic abortion. 2 months pregnant. N.P.N. 235 mg.%.	--	31.3

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	Age	Ergothioneine mg.%
Paroxysmal idiopathic haematuria. (3 years)	52	11.1
Ergothioneine range	6.7 - 31.3 mg.%	
Ergothioneine mean	14.8 mg.%	

21. Infections. Upper Respiratory

Acute pharyngitis.	34	7.6
Otitis media.	18	8.5
Hypertrophic tonsils.	26	8.9
Tonsillitis.	9	10.4
Chronic otitis media.	52	10.6
Acute pharyngitis.	35	12.6
Acute pharyngitis.	6	14.9
Acute pharyngitis and tonsillitis.	22	21.0
Tonsillitis.	29	26.8
Ergothioneine range	7.6 - 26.8 mg.%	
Ergothioneine mean	13.5 mg.%	

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22. Gynecological Surgical Conditions*

	Age	Ergothioneine mg.%
Endometrial hyperplasia. B.P. 180/100	50	8.2
Fibromyomata.	38	11.0
Chronic pelvic inflammatory disease. Endometriosis. Fibromyoma.	46	14.5
Corpus luteal cyst. Diabetic mother.	25	15.8
Prolapse.	35	18.4
Ergothioneine range	8.2 - 18.4 mg.%	
Ergothioneine mean	13.6 mg.%	

*All values given are on pre-operative specimens.

23. Dermatitis Eczema, and Urticaria

Chronic dermatitis.	67	6.0
Dermatitis.	60	9.0
Stasis dermatitis. Varicose veins. Pregnant 9 months	30	10.7
Eczema.	23	13.1

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	Age	Ergothioneine mg.%
Stasis dermatitis. B.P. 160/70	81	26.3
Urticaria.	51	16.4
Urticaria.	37	21.8
Ergothioneine range	6.0 - 26.3 mg.%	
Ergothioneine mean	14.8 mg.%	

A. Miscellaneous Diseases

Therapeutic abortion. (2 months pregnant) Rheumatic heart disease. Hydatidiform mole.	23	14.3
Vaginal bleeding of unknown cause.	--	8.4
Menopausal menorrhagia. (for 19 weeks)	45	16.7
Intestinal polypi. B.M.R. plus 27	58	10.9
Hemorrhoids. (for 20 years)	45	12.3
Faecal impaction (4 days) Osteoarthritis. Cataracts. B.P. 170/92.	78	15.3
Pancreatitis, acute. (sample taken 29 days later)	60	12.3

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	Age,	Ergothioneine mg.%
Addison's disease. Pneumonitis. B.M.R. plus 40 and 50.	69	3.3
Adenoma of the pancreas. Hyperinsulinism. Fasting blood sugar 94 mg.%. (sample taken 6 days after operation)	33	10.0
Hypopituitarism. Multiple ulcerations of the colon.	41	13.3
Intracranial lesion.	32	6.5
Porial cirrhosis. Chronic bronchitis. (sample taken 5 days after laparotomy)	63	6.6
Old eye injury. Symblepharon.	35	10.5
Supparative parotitis. Subacute pyelonephritis. Serum Protein 7.0 gm.%, Albumin 3.24 gm.%.	11	10.6
Polycythemia vera. Red blood count 6.76 million. Retinal and vitreous haemorrhages.	22	16.7
Destruction of D12 vertebra. Pott's disease?	--	7.0
Syphilitic aortitis, with ruptured aneurysm.	43	11.7
Laceration of scalp. Temperature 101°F.	16	5.4
Low back strain.	27	8.1
Torn medial meniscus. B.P. 150/90.	29	10.0

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	Age	Ergothioneine mg.%
Periarticular tear. B.P. 165/100	45	10.4
Sacro-iliac strain.	43	15.4
Low back strain. Old pelvic inflammatory disease with spotting (blood) and discharge.	32	30.5
No diagnosis. Gastro-intestinal symptoms.	33	3.5
Pylorospasm. B.P. 160/80.	66	5.4
Low back pain. Functional colon disorder.	32	6.0
Fever of unknown origin.	13	10.1

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